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Screening of cotton germplasm against cotton leaf curl virus disease (CLCuD) and role of weeds for its development

ALOK KUMAR, D. MONGA* AND KISHOR CHAND KUMHAR
Central Institute for Cotton Research, Regional Station, Sirsa-125 055
**E-mail: dmonga2009@gmail.com*

ABSTRACT : Cotton leaf curl virus disease (CLCuD) caused by single stranded gemini virus and transmitted by whitefly in persistent manner is an important disease in northern cotton growing region of India. The disease is characterized by upward/downward curling of leaves, the veins of the affected leaves become thickened which are most pronounced on the underside. Two types of veins thickenings are seen, small vein thickening and main vein thickening. Infected plants later develop leafy enations on the underside of leaf. In severe conditions, plants become stunted leading to reduction in yield. One hundred germplasm lines were screened at Central Institute for Cotton Research Regional Station, Sirsa during 2008-2009 and 2009-2010. Result based on 2 years data showed that out of 100 germplasm lines 20, 70 and 10 germplasm lines were resistant, moderately resistant and moderately susceptible, respectively.Detection of cotton leaf curl virus (CLCuV) in weeds showed that *Convulvu sarvensis (*Hirankhuri), *Spinacea* sp. *(*JungliPalak*), Solanum nigrum* (Blackberry nightshade), *Lantana camara* (Raimuniya), *Chenopodium album* (Bathua) were among naturally collected weeds which were detected positive by PCR amplification.*Achyrenthu saspera*(Puthkanda), *Digeriaavensis* (Tandla), *Croton sprucifera*(Junglimirch) and *Xanthium strumarium*(Gutpatna) were among artificially inoculated weeds which were found CLCuV positive by developing CLCuD type symptoms and by PCR amplification. Transmission of CLCuV from cotton to weeds and *vice versa* was also successful.

Key words : Amplification, CLCuV, CLCuD incidence, cotton, PCR, PDI, quality parameters, transmission, weeds

Cotton is the most important *kharif* cash crop of India. Among the various factors responsible for its low production and productivity in northern Indian states including Haryana, Punjab and Rajasthan, cotton leaf curl virus disease (CLCuD) has been found to be one of the major limiting factor. The disease is caused by a single stranded circular Gemini virus consisting of DNA- A and 2 satellites *i.e. alfa* and *beta* and is transmitted by whitefly (*Bemisia tabaci*). This disease is characterized by upward/ downward curling of leaves, the veins of the affected leaves become thickened which are most pronounced on the underside. Two types of veins thickenings are seen, small vein thickening and main vein thickening. Infected plants later develop leafy enations on the underside of leaf. In severe conditions, plants become stunted leading to severe yield reduction.Understanding of inoculum source is an important step in epidemiological studies. With the development of serodiagnostic and PCR based molecular tools, the quick and reliable detection of cotton leaf curl virus has become possible. Apart from cotton, the virus can infect several collateral and alternate hosts as well as weeds that act as source of inoculum for its spread from one season to the other by its vector *i.e.* whitefly. However, symptoms of the disease have not been properly defined on weeds. A weeds and other hosts harbour this virus in the off season and serve as the main source of primary inoculum for the development of disease on cotton in the next season. The disease migrates

from these plants to cotton crop through its carrier, whitefly. The main objective of the present study was to find out resistant material/ genetic source and to identify weed hosts carrying primary inoculum for cotton leaf curl virus disease development.

MATERIALS AND METHODS

Screening of germplasm lines : One hundred germplasm lines obtained from Director, Central Institute for Cotton Research, Nagpur were screened at experimental farm of Central Institute for Cotton Research, Regional Station, Sirsa for consecutive 2 years 2008-2009 and 2009-2010. Germplasm lines with infector rows of CLCuD susceptible variety HS6 after every 4 lines were sown in May in 3 replications in CLCuD screening nursery at CICR Research Farm with a spacing of 67.5 cm between line to line and 30 cm between plant to plant using randomized block design. Recommended agronomic practices were carried out from sowing to harvesting. No sprays were carried out for the control of sucking pests. All the plants of a genotype were thoroughly observed for appearance of cotton leaf curl virus symptoms such as vein reticulation, vein thickening, curling and cupping of leaves, leafy enations, shortening of internodes and stunting of plant etc. Data on cotton leaf curl virus disease incidence on 0-4 disease grade scale were taken (Table 1).The CLCuD Per cent Disease Index (PDI) was calculated for each entry by using following formula:

PDI = Average grade/Maximum grade x 100

Average grade was calculated with the help of following formula:

Average grade =Sum of all grades/Total plants

On the basis of PDI,germplasm lines were categorized resistant, moderately resistant, moderately susceptible and susceptible (Table 2).

Table 1. Disease rating scale and CLCuD symptoms

Rating scale	Disease symptoms
0	Plants completely free from leaf curl disease
1	Thickening of small vein (SVT), leaves/ veins appearing dark green, only few upper leaves affected
2	Thickening of veins, curling and cupping of leaves
3	Thickening of veins, curling and cupping of leaves, appearance of one to many leafy enations of different shapes and sizes on under side of leaves
4	Thickening of veins, curling and cupping of leaves, appearance of one to many leafy enations of different shapes and sizes on under side of leaves, stunting of plants leading to bushy appearance

At the time of crop maturity,data for seed cotton yield/plant (g) and quality parameters were recorded. For yield/plant cotton bolls of each plant were picked and weighed by weighing balance and/plant yield was noted. For determination of quality parameters such as fibre length (mm), uniformity ratio (UR), micronaire value (MIC) and tenacity (g/tex), 100 g lint samples of each germplasm line was submitted to CIRCOT unit, Sirsa and results were obtained.

Table 2. Per cent disease index (PDI) and reaction of germplasm against CLCuD

Reaction	Per cent Disease Index (PDI)
ImmuneResistant	00.1-5
Moderately Resistant	5.1-25
Moderately Susceptible	25.1-50
Susceptible	>50

Identification of weeds carrying CLCuV inoculum : A total of 339 weed samples (261 before sowing of cotton and 78 after cotton crop harvesting) of 35 different types were collected during present investigation from 2008 to 2010.CLCuV infected cotton plants were also collected from CICR, Regional Station, Sirsa experimental farm to use them as check for PCR amplification.

Weeds showing CLCuD type symptoms and harbouring whiteflies were collected from hot spots of Haryana, Punjab and Rajasthan during 2008 to 2010. Weeds were collected 2 times in a year before sowing of cotton crop and after harvesting of cotton crop. Collection was carried out during the month of April-May and December. In Haryana, leaf samples of weeds were collected from experimental farm CICR, Regional Station Sirsa. In Punjab, leaf samples of weeds were collected from experimental farm of Abohar and Faridkot and from farmer's field in Goniyana (Bathinda). In Rajasthan, leaf samples of weeds were collected from experimental farm of ARS, Sriganganagar. A minimum of 5-6 young leaves of weeds were plucked and collected in polybags. Polybags were then kept in a box containing dry ice and stored in deep freezer (Remi) at -70°C.

Genomic DNA isolation from weeds and cotton : Genomic DNA from all the weeds and cotton (used as CLCuD positive check) was isolated from collected young leaf samples using modified C-TAB (cetyltrimethyl ammonium bromide) method developed by Murray and Thompson (1980) and modified by Rogers and Bendich (1988).

Polymerase chain reaction (PCR) for detection of CLCuV from weeds and cotton : In the present study, coat protein (CP) primer pair was used for the detection of CLCuV in weeds and cotton.DNA isolated from leaf tissue of different weeds and infected cotton plants was subjected to amplification by PCR using CP -F and CP –R to check the presence of CLCuV. The nucleotide sequence of these primers are 5'-CGG GAT CCA TGT CGA AGC GAG CTG CC-3'and 5'-CCG GAA TTC ATA TCA ATT CGT TAC AGA GTC A -3' (Imperial Life Sciences).

For PCR amplification following reactants were used:

Genomic DNA (50ng	2 µl
CP primer (Forward) CP-F	1.5 µl
CP primer (Reverse) CP-R	1.5 µl
PCR Master Mix (1 X)	45 µl

50 µl reaction mixture was prepared for PCR by adding above mentioned reactants. The reactants were taken in PCR tube, carefully mixed and spun down briefly to collect the contents to the bottom of the tube. Amplification was performed in a Thermal cycler PTC-100 (M. J. Research Inc., USA) under the following conditions:

One cycle for initial denaturation at 95 ^{0}C for 4 min,29 cycles of denaturation at 94^{0}C for 30 sec, annealing at 55^{0}C for 30 sec. and extension at 72^{0}C for 45 sec. An additional cycle at 72^{O}C for 10 min. was run at the end of these cycles.

After PCR, the PCR products (10 µl) were resolved by 1 per cent agarose gel electrophoresis.

Transmission studies of CLCuV : This was assumed that cotton leaf curl virus (CLCuV) migrates from weeds to cotton during crop season and from cotton to weeds during off season by its vector whitefly in persistent manner. To confirm this assumption, weeds and cotton plants were raised in polyhouse in artificial conditions.

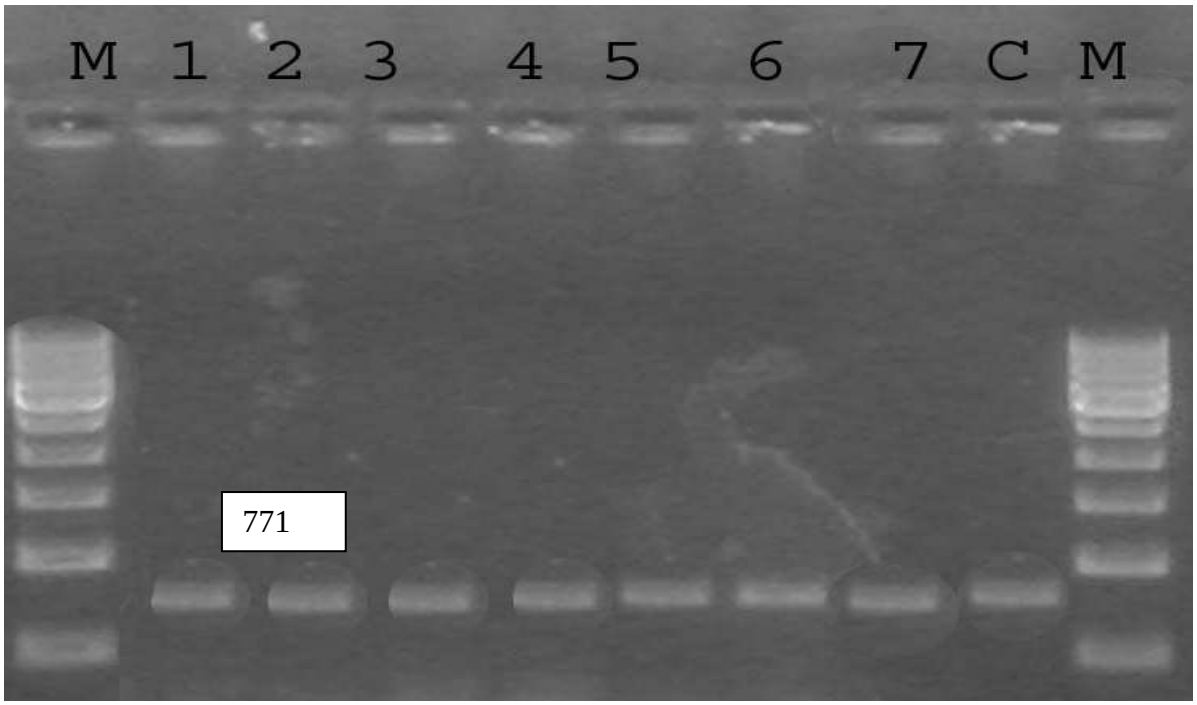

Fig. 1. Cotton germplasm DNA amplification: Lane M: 1 Kb Molecular weight marker, Lane 1-7 cotton DNA and Lane C: check

Table 3. Mean PDI and quality parameters of resistant germplasm lines during 2008-2009 and 2009-2010.

Germplasm lines	Mean					
	PDI	Yield/plant	2.5 per cent SL (mm)	UR	MIC	Tenacity (g/tex)
Green Wichita	1.91	12.58	26.6	51	4.5	20.3
FFS 47	4.86	45.38	26.6	49.5	4.5	19.6
SA 1150	0.0	47.23	26	46.5	4.5	20
SA 1006	2.5	19.84	26.7	45.5	4.5	19.8
DPEL 16	4.73	22.75	26.3	48.5	4.4	19.7
SA 1038	0.0	30.8	26.5	48.5	4.5	20.5
NCAC 9	2.81	39.81	25.5	50	4.3	20.2
NCAC 15	2.62	27.12	25.9	50	4.7	20.3
SA 1337	4.21	23.75	25.5	47	4.5	19.1
VC 17	0.0	15.14	25.9	49	4.4	17.5
SA 979	0.0	17.07	25.6	49	4.7	18.9
AKG 2/49	0.0	22.64	28.0	47	4.4	19.1
SA 112	0.0	26.8	25.6	50.5	4.9	18.9
A 02 N99	0.0	38.24	26.1	49	4.4	19.6
Cotton 14	3.66	17.14	25.5	52	4.7	19.4
A 02 N68	3.61	18.67	25.7	51	4.7	18.6
SA 1058	4.02	18.36	26.5	47.5	4.9	19.2
NCAC 7	2.65	19.31	26.1	51.5	4.3	19.6
SA 520	1.79	22.55	25.9	51	4.6	19.9
DP 45 A (4)	3.24	29.1	25.8	50.5	4.7	19.8

Transmission of CLCuD from cotton to weeds : To study transmission of white flies CLCuV from cotton to weeds, weeds were raised in polyhouse and then artificially inoculated by white flies carrying CLCuV inoculum acquired from infected cotton plants.Five seeds of each weedplant (Puthkanda-*Achyranthesaspera*, Congress grass *Partheniumspp*, JungliMirch-*Croton sprucifera*, Gutpatna-*Xanthium strumarium* and Tandla-*Digeriaarvensis*) were sown in each pot and the pots were kept in cages to keep them free from whitefly population.After establishment of weed plants, they were inoculated by viruliferous whiteflies. To obtain viruliferous whiteflies, healthy (virus free) whiteflies (*B. tabaci*) were collected from *Clitoriaternatea* (immune to begomoviruses) with the help of aspirator. Pure culture of whitefly population was checked by PCR for presence of CLCuV. After PCRdetection, whiteflies were fed on CLCuD infected cotton plants for 24 h for acquisition of virus. After acquisition period, 20 white flies were transferred to each weed plant and kept on them for 24 h After 24 h, white flies were removed by spraying appropriate insecticide. Weed plants were regularly observed for initiation of cotton leaf curl virus disease symptoms

DNA from artificially inoculated weed plants was isolated by CTAB method. DNA isolated from artificially inoculated weed plants was amplified by PCR to check the presence of CLCuV.After PCR, the PCR products (10 µl) were resolved by 1 per cent agarose gel electrophoresis.

Transmission of CLCuD from weeds to cotton : Same methodology was used as described above for this purpose and cotton plants were inoculated with viruliferous whiteflies which were fed on infected weeds for acquiring virus and later inoculated cotton plants were checked by PCR for presence of virus.

RESULTS AND DISCUSSION

During 2008, out of 100 germplasm lines 75 lines were found resistant and 25 lines were found moderately resistant while in 2009 12, 51, 31 and 6 germplasm lines were found resistant, moderately resistant, moderately susceptible and susceptible respectively. Graded per cent disease index (PDI) results based on 2 years study revealed that out of 100 germplasm lines 20, 70 and 10 germplasm lines were resistant, moderately resistant and moderately susceptible respectively. Seven germplasm lines were found free from CLCuV in field condition so they were checked for the presence of CLCuV by PCR. For this purpose, DNA from these free lines was isolated and amplified by PCR. PCR results showed that all lines were CLCuV positive showing an amplified product of 771 bp. It means that CLCuV was present in these free lines, but was not expressed which may be due to less inoculum or may be in latent form. PCR results are presented in Fig. 1.

Mean PDI for resistant lines ranged from 0.00 to 4.86 per cent. Maximum PDI in resistant lines was 4.86 in FFS 47 cotton germplasm followed by DPEL 16 (4.73), SA 1337 (4.21), SA 1058 (4.02) and cotton 14 (3.66). Mean yield/ plant for resistant germplasm lines ranged between 12.58 g to 47.23 g. Maximum mean yield/plant was observed in SA 1150 (47.23 g) followed by FFS 47 (45.38), NCAC 9 (39.81), A 02 N99 (38.24) and SA 1038 (30.8 g) (Table 3). Mean uniformity ratio (UR), mean 2.5 per cent span length (2.5% SL), mean micronaire value (MIC) and mean tenacity (g/tax) for resistant germplasm lines ranged between 45.5 to 52, 25.5 mm to 28.0 mm, 4.3 to 4.9 and 17.5 to 20.5,

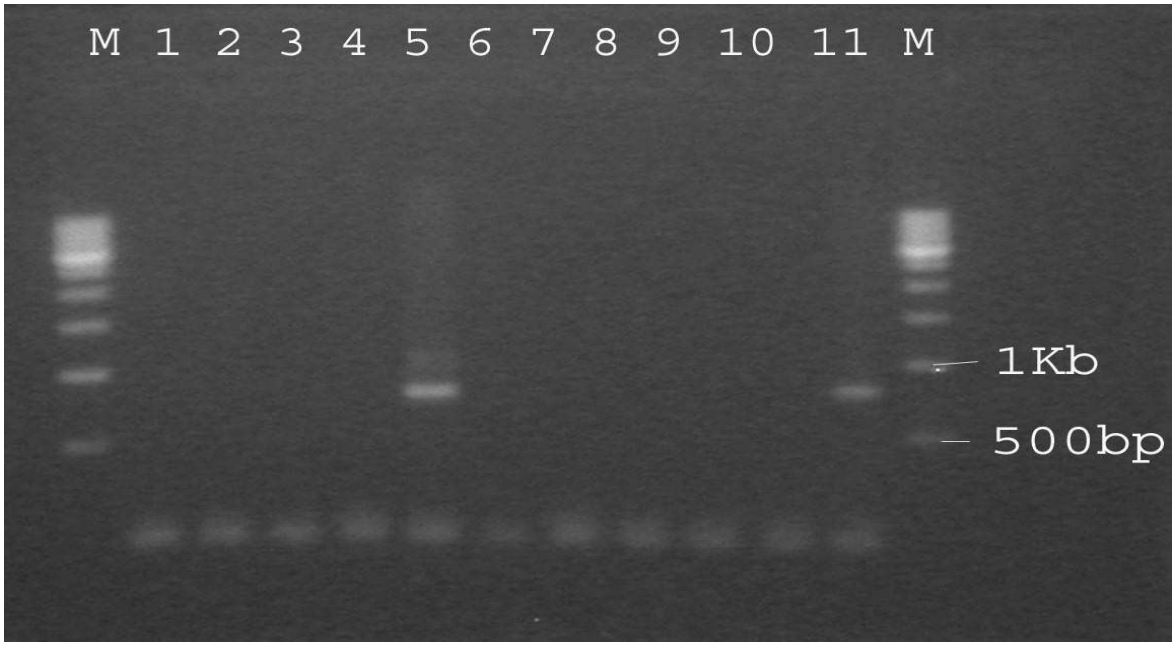

Fig. 1 PCR amplification of weeds DNA collected from Abohar- Fazilka road side. Lane M- 500 bp molecular marker Lane 1-10 weeds **(**1. *Partheniumspp*2.*Capsicum species* 3. *Acyrenthusaspera* 4.*Withaniasomanifera* 5.*Convulus arvensis* 6.*Chenopodiummurale*7.*Abutilon indicum*8.*Halianthusspp*9.*Aerucapersica*10.*Cannabis spp.***)**Lane11. *Check*

respectively (Table 3).

Mean PDI for moderately resistant lines ranged from 5.06 to 22.79 per cent. Minimum PDI in moderately resistant lines was observed in SA 223 (5.06%). Maximum PDI in moderately resistant lines was 22.79 in cotton germplasm line NA 1375 followed by Pusa 109/10 (22.74), SA 1031 (21.6), SA 12F (20.21) and SA 1178 (19.44). Mean yield/ plant for moderately resistant germplasm lines ranged between 8.9 (SA 1178) to 183.41 g. Maximum mean yield/ plant was observed in cotton 131 (183.41) followed by SA 1197 (138.8 g) FFS 76 (68.06), LK 861 (62.37), and SA 240 (53.86 gm). Mean uniformity ratio (UR), mean 2.5 per cent span length (2.5% SL), mean micronaire value (MIC) and mean tenacity (g/tex) for moderately resistant germplasm lines ranged between 47.5 to 52.5, 24.2 to 27.5 mm, 4.1 to 5.0 and 17.3 to 22.4, respectively.

Mean PDI for moderately susceptible lines ranged from 25.61 to 33.5 per cent. Minimum PDI was observed in SA 883. Maximum PDI in moderately susceptible lines was 33.5 per cent in cotton germplasm line TAM 87 N5 followed by AKG 2154 (32.01), SV 213 (30.87), SA 105 (30.8) and TNAU 35001 (30.72). Mean yield/ plant for moderately susceptible germplasm lines ranged between 17.81 to 36.82 gm. Minimum yield/plant was noticed in SA 105 (17.81 g). Maximum mean yield/plant was observed in SA 883 (36.82) followed by TNAU 35001 (33.71 g) VCA 5 (32.88), FFS 91 (29.96), and CNH 1025 (29.29 g). Mean uniformity ratio(UR), mean 2.5 per cent span length (2.5% SL), mean micronaire value (MIC) and mean tenacity (g/tex) for moderately susceptible germplasm lines ranged between 49.5 to 51.5, 24.0 to 27.6 mm, 4.2 to 5.1 and 17.7 to 21.4, respectively.

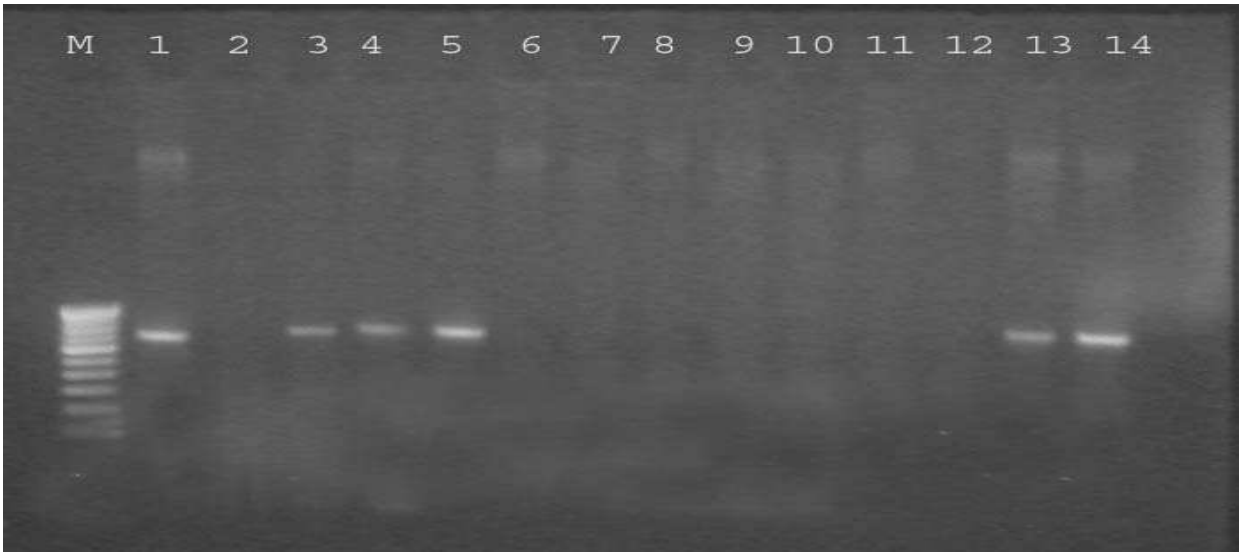

Fig. 2. PCR amplification of weeds DNA collected from CICR RS Sirsa. Lane M- 100 bp molecular marker, Lane **1.***Spinacea*sp.**Lane 2.***Hibiscus rosasinensis***Lane 3.** *Chenopodiumalbum* **Lane 4.***Solanumnigrum* **Lane 5.** *Lantana camara***Lane 6***Solanumtuberosum* **Lane 7.** *Ageratum conyzoides***Lane 8.***Partheniums*p**. Lane 9.***Croton sprucifera* **Lane 10.***Achyeanthesaspera* **Lane 11.***Withaniasomnifera.***Lane 12.***Aerucasativa* **Lane 13.***Check* **Lane14.***check*

Lines resistant to cotton leaf curl virus disease have been identified by previous workersalso (Ajmara*et al.,* 2004,Rashida*et al.*, 2005,Radhakrishnan*et al.*, 2004, Monga*et al.,* 2008, Ahmad *et al.,* 2010).It was important to note that yield/ plant in resistant lines ranged from 12.58 to 47.23 g where as in case of moderately resistant germplasm lines it was 8.9 to 183.4 g/ plant. There were 5 entries namely Cotton 131, SA 1197, FFS 76, LK 861 and SA 240 where yield was more than 47.23 g/plant *i.e.* highest yield noted in the case of resistant lines and their mean PDI ranged from 5.82 to 18.75. The result showed that moderately resistant lines may show much higher yield than the resistant lines due to their inherent genetic potential. Such lines can also be considered as varieties or sources for hybridization programme.

Fig. 3. *Croton sprucifera* ,*Achyranthesaspera* and *Digeriaarvensis* showing CLCuD symptoms

Detection of CLCuV in weeds collected from hot spots by PCR amplification : There was no amplification with primers specific to coat protein, indicating that CLCuV was not present in weeds collected during April 2008 and April 2009 from hot spots of Haryana, Punjab and Rajasthan. Among the weeds collected during December, 2008 and 2009 from CICR Sirsa farm, AboharFazilka road side and Sriganganagar-Abohar road side, only one weed, namely *Convolvusarvensis* (collected from AboharFazilka road side during 2008) showed CLCuV presence by showing amplification of the expected 771 bp size PCR product (Fig. 1).

Four weeds collected during December, 2010 from CICR Sirsa farm out of 12 weeds, namely *Spinacea*sp. *(*JungliPalak*), Chenopodium album*(Bathua), *Solanumnigrum*(Blackberry nightshade)*, and Lantana camara*showed positive response towards CLCuD detection by amplifying a product of the expected 771 bp size (Fig. 2).

Thus, out of 339 samples of 35 different types of weeds collected during 2008, 2009 and 2010, only 5 weeds namely *Convulvusarvensis (*Hirankhuri) collected from Abohar- Fazilka Road Side (December, 2008), *Spinacea*sp. *(*JungliPalak*), Solanumnigrum*(Blackberry nightshade)*, Lantana camara*and *Chenopodium album*(Bathua)*,* (collected from CICR Sirsa December, 2010)showed positive response towards CLCuD detection by amplifying a product of expected 771 bp size.

CLCuD transmission from cotton to weeds

Detection of CLCuV in artificially inoculated weeds in polyhouse : Out of 5 artificially inoculated weeds in poly house, 3 weeds namely *Achyranthesaspera*(Puthkanda), *Digeriaarvensis* (Tandla) and *Croton sprucifera*(Junglimirch) showed CLCuD symptoms (Fig. 3).

In PCR amplification, out of 5 weeds 4 weeds namely *Achyrenthesaspera* (Puthkanda), *Digeriaarvensis* (Tandla), *Croton sprucifera*(Junglimirch) and*Xanthium strumarium*(Gutpatna)showed a positive response towards CLCuD detection (Fig. 4).

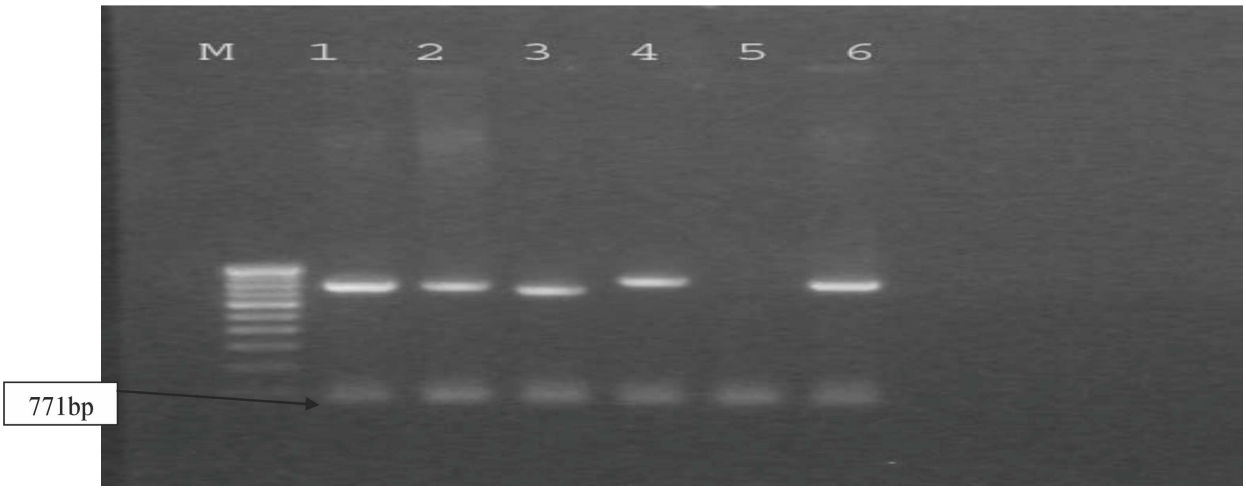

Fig. 4. PCR amplification of DNA of weeds inoculated artificially in Polyhouse. Lane M- 100 bp molecular marker; **Lane 1.***Achyrenthusaspera* **Lane 2.***Digeriaarvensis* **Lane3.***Croton sprucifera Lane* **4.***Xanthium strumarium***Lane 5.***Partheniums*p.**Lane 6** *Check*

Fig. 5. Transmission of CLCuV from weeds to cotton: Cotton plants artificially inoculated by whiteflies carrying inoculum from CLCuV infected weeds.

Thus, out of 5 weeds inoculated artificially in polyhouse 4 weeds were detected CLCuV positive by PCR amplification from which 3 weeds showed CLCuD symptoms after 30 days of inoculation.

CLCuD transmission from weeds to cotton

Detection of CLCuV in artificially inoculated cotton in ploy house : In polyhouse cotton plants were artificially inoculated using whiteflies where virus was acquired from weed plants (*Croton sprucifera*). The development of CLCuD symptoms started after 20 days of inoculation and all the plants showed the CLCuD symptoms (Fig. 5). Presence of CLCuV was confirmed through PCR.

Artificially inoculated cotton plants by whiteflies collected from CLCuV infected weeds showing CLCuD symptoms : Thus, it was confirmed that CLCuVis transmitted from cotton to weeds during off season and from weeds to cotton during crop season by its vector whitefly.

A large weed hosts carrying CLCuV inoculum have been reported by previous workers alsoon the basis of visual symptoms, transmission studies and ELISA,DNA-A probe hybridization, presence of DNA-A and Beta DNA of CLCuV,DNA-A and DNA beta probe hybridization andPCR using CP primer (Sharma and Rishi, 2003, Sivalingam *et al.,* 2004; Radhakrishnan*et al.*, 2004, Kang *et al.*, 2004 and Monga *et al.*, 2004)

The present study helped in identifying

weeds as inoculum source of CLCuD and will be helpful in management of this important disease by timely roughing of weed plants carrying this virus in off season to prevent further spread of this virus.

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